THERMAL RESISTANCE OF BACILLUS LARVAE SPORES IN HONEY[1]

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Considerable attention has been given to the effect of heating in various media on the viability and virulence of the spores of *Bacillus larvae*, the causative agent of the honeybee disease, American foul brood (White, 1920). Few data have been reported, however, concerning temperatures above the boiling point of honey (White, 1920; Burnside, 1940*a*, 1945). Under a project on sterilization of honey to permit feeding it to bees without danger of spreading disease, thermal death-time studies were conducted on spores of this organism in diluted honey over a temperature range of 100 to 140 C. The results reported here have been used in the development of a process for treatment of honey to permit feeding it to bees without danger of transmission of American foul brood.

Although Burnside (1945) reported that "boiling for 30 minutes can be depended upon to destroy the virulence of *Bacillus larvae* under ordinary conditions", the authors have been advised (Hambleton, 1950) that any processing of honey for bee feeding must guarantee absence of viable spores of this organism, not simply destruction of virulence. Burnside (1940*a*) has reviewed the literature on the heat resistance of *B. larvae* spores and investigated the thermal resistance of the spores in diluted honey, water, and beeswax and in the dry state. He reported growth in a culture of spores boiled in water or exposed to flowing steam for 7 hours, boiled for 5 hours in 50 per cent honey, autoclaved in water at 15 pounds per square inch for 25 minutes or dry for 40 minutes, exposed to 98 C dry heat for 2 days or in beeswax for 5 days at 100 C. He stressed the importance of heavy inocula in thermal death studies and the use of media favorable to the germination of heated spores.

METHODS

Spore suspension. B. larvae spores were obtained from infected honeycombs from Wyoming by picking suitable numbers of scales (naturally dried honeybee larvae killed by foul brood) from the comb. They were disintegrated by shaking with glass beads in sterile water at a concentration of 4 scales per milliliter. After being strained through cheesecloth, the spore suspension was counted microscopically. Prepared in this manner, the suspension contained 8.42×10^9 spores per milliliter.

To 10.752 kg of buckwheat honey were added 105 milliliters of this suspension. The mixture was stirred for 5 hours to obtain uniform dispersion. This suspen-

[1] Report of a study made under the provision of the Research and Marketing Act of 1946.

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sion of *B. larvae* spores in honey was used for the work reported below. It was calculated to contain 115×10^6 spores per milliliter and found by counting at various times to contain 100 to 150×10^6 spores per milliliter.

An electrically heated, agitated oil bath was used, thermostatically controlled at the desired temperature within ±0.4 C. Standard pyrex thermal death-time (TDT) tubes, described by Bigelow and Esty (1920), were used; a wire rack was employed to immerse the tubes in the bath.

The spore-containing honey was diluted immediately before use to 55 per cent solids and acidified[3] with dilute H_3PO_4 to pH 3.0, producing a final acid concentration of 0.2 per cent. The honey was pipetted in 2.3-ml portions into sterile TDT tubes, which were then sealed. Five such tubes were used for each time-temperature relationship. Tubes were heated by immersion in the oil bath at 100, 110, 121.5, and 132 C for various times.

Come-up time correction. A correction of 3 minutes and 20 seconds for time required for the tubes to reach the temperature of the bath was originally made, as suggested by Ball and reported by Sognefest and Benjamin (1944), for TDT tubes containing 60 per cent sugar solution held stationary in an oil bath. After the data were obtained, an approximate "z" value of 26 for the TDT curve ("z" = degrees F traversed by the curve in one logarithmic cycle) was calculated. Sognefest and Benjamin (1944) show corrections of come-up times for TDT tubes containing 60 per cent sugar heated in steam using "z" values of 14, 18, and 22. These data were extrapolated to "z" = 26, yielding a value of 0.95 minutes. This was then multiplied by Ball's oil bath to steam time ratios of 3.30/1.30, yielding a final come-up time correction of 2 minutes and 25 seconds. The Sognefest and Benjamin values take account of the lethality of the come-up time. Desrosier and Esselen (1951) report that upon heating standard TDT tubes in an oil bath over the range of 225 to 250 C, "a previously determined correction of 2.3 minutes was allowed for the lag period". Small differences in lag time are important only when the heating time is relatively short. The values plotted in figure 1 are the total time in the bath less 2 minutes and 25 seconds.

After heating, the tubes were immediately immersed in ice water, cooling in a few seconds. When there was insufficient time to culture the samples immediately, the tubes were refrigerated overnight and brought to room temperature the following morning. The tubes were opened, and 2.0 milliliters of honey were pipetted into a sterile 50-milliliter centrifuge tube. Twelve milliliters of sterile distilled water were added, and the tubes were centrifuged for 1 hour at a RCF of 850. The supernatant was replaced with 15 milliliters of the medium recommended by Katznelson and Lochhead (1944), and the tubes were incubated at 37 C. After 3 to 5 days, each culture was tested for the presence of nitrites, a presumptive test for *B. larvae* under these conditions (Sturtevant, 1932; Burnside, 1940*b*). All positive tubes were further examined microscopically for growth, with subculture if necessary to identify the organism. Tubes were incubated for

[3] Acidification to pH 3 was necessary to prevent a heat-caused precipitation in buckwheat honey.

30 days before being discarded as negative. It was found that control TDT tubes contained more than 10^7 viable spores per milliliter.

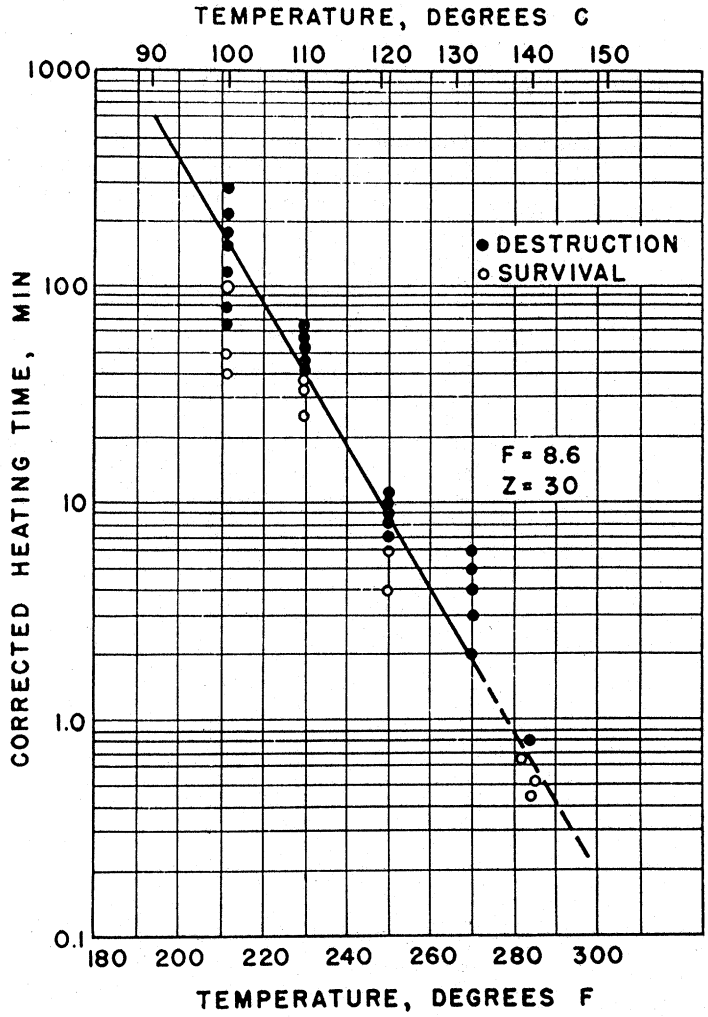

Figure 1. Thermal resistance of *Bacillus larvae* spores in honey.

RESULTS

The results are plotted in figure 1. The procedure outlined by Townsend, Esty, and Baselt (1938) was followed in establishing the curve; its semilogarithmic nature is evident. The figure shows that exposure to a temperature of 100 C for 160 minutes, 110 C (230 F) for 41 minutes, 121 C (250 F) for 8.67 minutes, or 132 C (270 F) for 1.9 minutes reduced the viable *B. larvae* spore population from 10^7 per milliliter to less than 1 per 10 milliliters in dilute honey at pH 3.

The line in figure 1 has an "F" value of 8.6 (minutes at 250 F for destruction) and a "z" value of 30. These two constants permit reproduction of the curve.

Continuous sterilization process. The points shown on the figure near 285 F (140 C) were determined by a procedure different from that previously described. A continuous flow, high temperature, short time heating unit was constructed by modification of existing equipment. Employment of a variable delivery pump and several thermocouples made it possible to obtain accurate time-temperature data on its operation.

A spore suspension was prepared from infected combs by disintegration of 68 g of whole comb in 1,000 ml of water in a Waring blendor, followed by straining through cheesecloth. The resulting spore suspension contained 2.32×10^9 spores

TABLE 1

Continuous process heating of honey containing Bacillus larvae spores

RUN NO.	INOCULUM	PREHEAT		HOLD		RATE OF FLOW PER HOUR	NO. POSITIVE SUBCULTURES
		Time	Temp	Time	Temp		
	spores per ml	*sec*	*C*	*sec*	*C*	*gal*	
1	140×10^6	2.0	138.3	40	138.9	25	1 of 10
2	140×10^6	1.4	138.9	27	140.0	37	4 of 10
3	190×10^6	2.4	138.9	49	140.0	20	0 of 10
4	190×10^6	1.6	139.5	32	140.5	31	1 of 10

TABLE 2

Thermal resistance of Bacillus larvae strains in dilute honey at 110 C

SOURCE	SURVIVAL	DESTRUCTION
	min	*min*
Wyoming	37	41
Ohio	32	36
Florida	<25	25
Utah	32	36
Wisconsin	<25	25

per milliliter. To 19.303 kg of fall flower honey were added 920 ml of the spore suspension, followed by 5 hours' stirring. The honey was found to contain 140×10^6 spores per milliliter, and showed growth of *B. larvae* in culture at the 10^{-8} dilution. It was diluted to 54 per cent solids and acidified to pH 3.0 with phosphoric acid. This solution was pumped through the heating unit, which was operating at the desired temperature and pumping rate on dilute honey. The spore-containing honey was passed through the unit at a rate of 25 gallons per hour, which was calculated to heat the material to 138 to 140 C in 2 seconds and provide 40 seconds holding time at that temperature. The superheated material was released through a valve to a vapor-liquid separator at atmospheric pressure, instantly cooling it to the boiling point, and the vapor produced was separated from the liquid stream. Samples of the hot liquid were taken from a

T-tube for culturing tests. After the samples were taken, the pumping rate was increased to 37 gallons per hour, corresponding to a 1.4-second come-up time and a 27-second holding time at 140 C. Samples were taken under these conditions and cultured.

Another similar experiment was conducted in which the times of heat exposure were different. Table 1 shows the results and operating data. Ten 5-ml subsamples from each run were cultured; of these growth was shown in the following: run 1, 1; run 2, 4; run 3, 0; and run 4, 1. These points, shown in figure 1, serve to substantiate the extrapolation of the TDT curve to this temperature.

Thermal resistance of several strains of B. larvae spores. The thermal resistance of four additional strains of *B. larvae* spores was determined by the tube method at 121 C. The strains originated in Florida, Ohio, Utah, and Wisconsin. The spore concentration in each case was adjusted to the same level as used for the Wyoming spores. As may be seen in table 2, none of these strains showed thermal resistance greater than that of the Wyoming strain (figure 1). In view of the degree of variation encountered among strains in this respect, it is possible that strains exist with greater thermal resistance.

Effect of bactericidal agents on B. larvae spores in honey. Before these TDT studies were undertaken, some effort was made to find a material which would be toxic to *B. larvae* spores in honey, either alone or in conjunction with heat treatment. A series of compounds was tested in honey, both alone and with autoclaving at 15 pounds per square inch for 5 to 30 minutes. After treatment, the spores were centrifuged from the honey and washed twice with water to eliminate any effect of residual material on subsequent culture tests. The materials tested and the maximum concentrations (in per cent) were: sodium benzoate, 1; sodium sulfite, 1; hexylresorcinol, 0.1; iodine, 0.5; propylene oxide, 2; subtilin,[4] 0.1; sodium dehydroacetate,[5] 0.1; neomycin,[6] 0.1; and the following quaternary ammonium salts[7] at concentrations up to 1 per cent; Hyamine 1622,[8] Hyamine 2389,[8] BTC,[9] Tetrosan,[9] LPT-927-1E,[9] Roccal, and Emulsept.

None of these compounds was completely effective as a sporicidal agent against *B. larvae* spores in honey, although propylene oxide effected a marked reduction in spore population without autoclaving, and the first, third, and fourth quaternary salts listed permitted reduction of autoclaving time at 15 pounds per square inch from 25 to 10 minutes at 0.1 per cent concentration to kill about 10^8 spores per milliliter in honey. At this level, however, the quaternary compounds proved too toxic to honeybees for practical use in this manner.

Katznelson (1950) has discussed the influence of various antibiotics and sulfa drugs on *B. larvae*, primarily the vegetative form, both *in vivo* and *in vitro*. He

[4] Supplied through the courtesy of Merck and Co., Rahway, New Jersey.

[5] Supplied through the courtesy of Dow Chemical Co., Midland, Michigan.

[6] Supplied through the courtesy of Upjohn Co., Kalamazoo, Michigan.

[7] Mention of trade names does not imply indorsement of such products by the Department of Agriculture over similar products not mentioned.

[8] Supplied through the courtesy of Rohm and Haas Co., Philadelphia, Pennsylvania.

[9] Supplied through the courtesy of the Onyx Oil and Chemical Co., Jersey City, New Jersey.

was unable to demonstrate any sporicidal effect of sodium sulfathiazole solutions, of clavacin, or a germicide, "amosol".

DISCUSSION

Much of the honey that might be used for bee feeding after suitable sterilization would be honey of pronounced flavor with limited commercial acceptance. Buckwheat honey is in this category. When heated, this honey forms a gelatinous precipitate, which could interfere with processing and possibly with later use. By acidification of such honey from its normal pH of 3.6 to 4.0 to pH 3.0, this coagulation can be avoided.

It is realized that in view of the logarithmic order of death of bacterial spores, there is no such thing as an absolute end point for their destruction (Stumbo, 1949). Several factors, however, render it extremely improbable that honey treated by a process based on these data could transmit American foul brood in bee colonies fed such honey. The number of spores with which the data were obtained (10^7 to 10^8 viable spores per milliliter) is much greater than the highest number reported to occur in honey produced by severely diseased colonies. Sturtevant (1951) determined the *B. larvae* spore content of samples of surplus honey taken from 290 combs produced in supers above excluders by 8 colonies having varying degrees of American foul brood infection. The highest number of spores (total viability unknown) in a sample of the surplus honey from a fairly heavily diseased colony was 46.2×10^4 per cubic centimeter. In practice, dilution with uninfected honey would reduce this considerably. Sturtevant (1932) has reported that at least 50×10^3 spores per milliliter of (sugar) sirup are necessary to infect a healthy colony with American foul brood. As pointed out by Woodrow (1941) and Burnside (1945), bee larvae more than $2\frac{1}{4}$ days old do not become infected with foul brood; hence spores with a heat induced delayed germination time of greater than $2\frac{1}{4}$ days are not virulent. The first effect of heating is slight delay in germination; more severe heating may cause a delay of several weeks (Burnside, 1940*a*, 1945).

In consideration of these factors, it is believed that honey given heat treatment according to the data presented herein will not transmit American foul brood if fed to honeybees. Toxicity tests and feeding tests of American foul brood infected honey sterilized by the continuous process mentioned before are in progress.

ACKNOWLEDGMENTS

The authors are indebted to James I. Hambleton of the Division of Bee Culture, Bureau of Entomology and Plant Quarantine, for the foul brood-infected combs and for toxicity and acceptance testing of various samples of honey. They also gratefully acknowledge the cooperation of J. B. Claffey, R. P. Homiller, and E. O. Strolle of the Chemical Engineering and Development Division of this Laboratory in designing, constructing, and operating the high temperature-short time heating equipment. Valuable counsel from Dr. H. Katznelson, Dr. S. R. Dutky, and Dr. T. C. Cordon is also appreciated.

SUMMARY

Thermal death-time studies were made on spores of *Bacillus larvae*. In dilute honey at a pH of 3.0 over a temperature range of 100 to 140 C, the thermal death-time curve obtained had a "z" value of 30 F and an F value of 8.6. Exposure of 10^8 spores per milliliter of honey of 55 per cent solids at pH 3 to the following conditions reduced viable spore population to less than 0.1 per milliliter: 160 min at 100 C, 41 min at 110 C, 8.6 min at 121 C, 1.9 min at 132 C, and 0.64 min at 140 C. A continuous method for sterilization of honey with respect to *B. larvae* spores has been based on these data.

Addition of the following compounds to dilute honey at the maximum concentrations (in per cent) listed did not destroy *B. larvae* spores in honey, either alone or in conjunction with 10 minutes' autoclaving at 15 pounds per square inch: sodium benzoate, 1; sodium sulfite, 1; hexylresorcinol, 0.1; iodine, 0.5; propylene oxide, 2; subtilin, 0.1; sodium dehydroacetate, 0.1; neomycin, 0.1. Three of four quaternary ammonium compounds tested at 0.1 per cent in conjunction with the heat treatment described above destroyed the spores but were too toxic to honeybees for practical use.

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